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Application of the Short-Term Chronic Test with *Ceriodaphnia dubia* in Identifying Sources of Toxicity in Industrial Wastewaters

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Effluents, whether from municipal or industrial sources, contain thousands of constituents which potentially may cause toxic effects to aquatic organisms. The toxicity of effluents to aquatic organisms is measured by standard methodology (EPA 1985; EPA 1989). Although long recognized as a rapid technique to measure toxicity of wastewaters, acute and short-term chronic testing provides a starting point for the hazard assessment of discharged wastewaters. To assist in separation and identification of toxicity in wastewaters the toxicity identification evaluation (TIE) program was developed. Identifying the source of toxicity in wastewater is based on the principal of sequential removal of the constituents coupled with a toxicity test of the fractionated effluent. The TIE programs conducted extensively over the past nine years used exclusively the acute toxicity test methods (Walsh and Garnas 1983; Gasith et al. 1988; Doi and Grothe 1989; Durhan et al. 1990; Ankley et al. 1990; Norberg-King et al. 1991; Jop et al. 1991a and b; Ankley et al. 1991). The acute TIE procedures were not effective with samples exhibiting marginal toxicity, therefore the Environmental Protection Agency (EPA) recently developed a methodology to identify chronically toxic constituents in wastewaters (EPA 1991). The general approach to the chronic TIE is similar to the acute methods, but the toxicity is defined using the short-term chronic methods.

The overall objective of this study was to identify and characterize the toxic constituents in two process waters which proved chronically toxic to the daphnid *Ceriodaphnia dubia* during the discharge toxicity evaluation program.

MATERIALS AND METHODS

Two wastewater samples, a landfill leachate treated in an experimental pilot plant (Effluent No.1) and an effluent from metal a finishing plant (Effluent No. 2) were used for the fractionation procedures. The identification of a toxic effluent fraction was based on the principal of sequential removal of a chemical fraction coupled with short-term (7-day), static-renewal toxicity tests with *Ceriodaphnia dubia* of the fractionated wastewater. The TIE procedures were conducted according to Jop et al. (1991b). Fractionation treatments used for identification of toxic compounds

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were used.

RESULTS AND DISCUSSION

Prior to initiation of the TIE program, chemical analyses and a short-term chronic test with effluent No.1 were performed using *Ceriodaphnia dubia*. The results of the short-term chronic test indicated that survival of *C. dubia* was not affected in any test concentration, while reproduction of daphnids expressed as number of offspring produced per female in the whole sample (100%) was significantly lower compared to the reproduction of control organisms (Table 1). The NOEC based on reproduction was 50%. Chemical analyses included assessment for inorganics, selected metals and volatile organics. The analysis revealed no detectable amounts of organics and metals but relatively high concentrations of calcium (360 mg/L), magnesium (160 mg/L) and sodium (110 mg/L). The high concentration of divalent ions resulted in relatively high hardness (1,920 mg $CaCO_3$/L) of this wastewater. None of the analyzed parameters by itself exceeded the threshold chronic concentration for *Ceriodaphnia dubia*.

Toxicity of a pressure-filtered, XAD, EDTA and nitrogen purge fractions were similar, and did not differ substantially from the toxicity observed in the whole effluent, with the exception of a nitrogen purge fraction in which *C. dubia* survival in 100% effluent was significantly different from the control (Table 1). The NOEC for these tests was 50%. Subsequently, two major cations (Ca and Mg) were partially removed from the effluent using clinoptilolite column. The total hardness of the eluate from the clinoptilolite column decreased to 220 mg $CaCO_3$/L. The clinoptilolite column reduced the concentration of calcium to 35 mg/L and magnesium to 42 mg/L, but increased sodium concentration to 790 mg/L. Although the NOEC value for the eluate from the clinoptilolite column was identical to that in the whole sample, *C. dubia* reproduction increased from the average of 4 offspring in 100% of the whole, filtered, nitrogen-purge, EDTA, and XAD fractions to 14 in the clinoptilolite fraction (Table 1). The elevated concentrations of calcium and magnesium which initially inhibited daphnid reproduction were substituted by sodium ions which affected *C. dubia* reproduction.

The results from the short-term chronic test with effluent No.2 indicated that survival of *C. dubia* was not affected in any test concentration, whereas reproduction of daphnids expressed as number of offspring produced per female in the whole sample was significantly lower compared to the reproduction of control organisms. The results of chemical analysis revealed 105 mg/L of calcium, 236 mg/L of sodium, 2.1 mg/L of magnesium, 3.9 mg/L of potassium and 30 μg/L of copper. Toxicity was removed after pressure-filtration, chelation with EDTA and XAD resin. In those fractions the reproduction of *C. dubia* increased from the 13-14 offspring in the whole sample to 16-19. Copper was identified as a potential source of chronic toxicity. Subsequently, metals were removed from the whole sample by cation-resin and C18 column and the eluates from both columns were tested. The eluate from these fractions were more toxic to *C. dubia* than the whole sample, indicating introduction of the artificial toxicity. The confirmation of the effluent No.2 toxicity was conducted by a spiking experiment in which toxicity increased in proportion to the increase of copper concentration. Addition of 10 μg of copper to the whole sample (final measured concentration was 38 μg Cu/L) include filtration, XAD-resin, nitrogen purging, EDTA, sodium thiosulfate, clinoptilolite and spiking treatments. Corresponding blanks of each fraction were also tested for toxicity to ensure that sample manipulation did not introduce artificial toxicity. Preliminary results from short-term toxicity tests with *C. dubia* indicated that biological response to toxicant(s) present in both effluents was not well pronounced (toxic response was defined as an inhibition of survival and/or reproduction in the whole sample), therefore, a standard 7-day short-term chronic test (EPA 1989) was used. The 7-day test is more sensitive then its abbreviated version, the 4-day chronic test (Masters et al. 1991) which was suggested by EPA-Duluth as an appropriate test method to use for the chronic TIE program.

Ceriodaphnia dubia (≤ 24 hours old) were used as the test organisms. The culture system consisted of three hundred 30-mL plastic beakers each containing 15 mL of culture medium and one adult. Daphnids were cultured in aged, hard well water with hardness raised by the addition of reagent grade chemicals according to the EPA method for hard water (EPA 1985). *Ceriodaphnia dubia* cultures were fed suspensions of a mixture of unicellular green algae (*Selenastrum capricornutum*) and YCT (yeast, Trout Chow and Ceriophyl) once a day. Twenty-four hours before test initiation, all immature daphnids were removed from the culture beakers. Offspring produced over an 8-hour period were culled individually using a glass pipet.

Samples were analyzed for the following parameters: inorganics (total cyanide, total phosphorus, total residual chlorine, chloride, ammonia, nitrate, alkalinity, hardness), organics (total organic carbon, organic nitrogen, anthracene, benzidine, chrysene, dichlorophenol, pentachlorophenol, phenol), metals (aluminum, cadmium, chromium total and hexavalent, calcium, magnesium, potassium, sodium, copper, iron, lead, nickel, silver, titanium, zinc) and volatile organic compounds (benzene, bromoform, bromomethane, carbon tetrachloride, chlorobenzene, chloroform, chloromethane, dibromochloromethane, 1,1-dichloroethane, 1,2-dichloroethane, methylene chloride, 1,1-dichloroethene, ethylbenzene, 1,1,2,2-tetrachloroethane, tetrachloroethylene, toluene, 1,1,1-trichloroethane, 1,1,2-trichloroethane, trichloroethylene, vinyl chloride, chloroethane, 2-chloroethyl vinyl ether, 1,2-dichloropropane).

The No Observed Effect Concentration (NOEC), which is the highest concentration that has no statistically significant adverse effect on the test organisms as compared with the control, and the Lowest Observed Effect Concentration (LOEC), which is the lowest concentration that has a statistically significant adverse effect on the test organisms as compared with the control, were determined using Fisher's Exact test (survival) and an analysis of variance followed by Williams, Dunnett's, Bonferroni's T-test, Steel's Many-One Rank Test or Kruskall-Wallis Tests (reproduction). The assumption that observations are normally distributed was validated before subsequent analyses (i.e., multiple comparison test). The Chi-Square Goodness of Fit Test was used to compare the observed sample distribution with normal distribution. As a check on the assumption of homogeneity of variance implicit in parametric statistics, data for each endpoint were analyzed using Bartlett's Test. To determine which of the test concentrations produced a statistically significant effect, multiple comparison tests

Table 1. Summary of the short-term static renewal toxicity tests on *Ceriodaphnia dubia* various effluent fractions.

Fraction	Effluent Concentration (%)	Effluent No.1 Survival (%)	Effluent No.1 Mean Number of offspring	Effluent No.2 Survival (%)	Effluent No.2 Mean Number of offspring
Whole	Control	100	23	80	26
	50	100	19	70	25
	100	100	6 **	70	14 ***
Filtered	Control	90	38	90	21
	Blank	90	25	90	16
	50	90	26	90	18
	100	70	1 **	90	19
XAD	Control	100	25	90	21
	Blank	90	18	90	16
	50	100	21	50	14
	100	80	5 ***	70	16
Na_2EDTA	Control	90	16	100	26
	Blank	100	18	100	21
	50	100	20	100	20
	100	80	5 ***	90	17
Nitrogen Purge	Control	100	37	100	23
	50	100	28	90	19
	100	50 *	3 **	80	5 ***
Clinoptilolite	Control	100	20		
	Blank	80	11		
	50	100	18		
	100	100	14 ***		
Sodium Thiosulfate	Control				22
	Blank			60	1
	50			80	13 ***
	100			100	8 ***

* Significantly different from the controls, according to the Fisher's Exact Test
* * Significantly different from the controls, according to Steel's Test
* * * Significantly different from the controls, according to Dunnets Test

decreased reproduction from 13-14 mean offspring per female in the whole sample to 8, while addition of 20 μg of copper (final measured concentration was 46 μg/L) resulted in increased mortality (60%) and inhibition of reproduction (3 offspring/female).

Effects of chemicals on aquatic organisms depend on the interaction of physical, chemical and biological factors as well as the duration of exposure. The relative importance of these factors will vary depending upon the organism, inherent properties of the chemical and interactions between the chemical and other ions present in water. Although ions like calcium or sodium, which are physiologically essential for freshwater species, may cause toxicity to these organisms when present in large quantities, e.g., effluent No.1. A freshwater invertebrate like *Ceriodaphnia* may lack the osmoregulatory capacity to maintain cellular ionic conditions in the presence of increased concentrations of the divalent or monovalent cations or in the absence of these ions. For example, sodium ions play a very important role in the osmoregulation of freshwater species by controlling the permeability of cell membranes to other ions. Therefore, concentration of both divalent and monovalent ions and their ratio in aquatic systems play a significant role in biochemical processes of various species.

Difficulties encountered during the chronic TIE program are a combination of inadequate fractionation methodology and limited choice of applicable test methods. In a recently published manual (EPA 1991) the authors suggest that the TIE program with freshwater effluents should be conducted using the species which detected the toxicity triggering the TIE. When an alternative species is chosen, it must be proven that this species is impacted by the same toxicant(s). Two species, *C.dubia* and *P. promelas*, currently are available for the chronic TIE program. A third species, *Selenastrum capricornutum,* included in the EPA chronic manual is not sensitive, while abbreviated chronic tests with *Daphnia pulex* or *D. magna* are not popular and their toxicological data base is practically nonexistent. The majority of wastewaters produce a stronger response to invertebrates than to fish (with the exception of some surfactants, ammonia and cyanide). Likewise, fish short-term tests require much larger sample volumes for testing. Therefore, for the most chronic TIE's, *C. dubia* is at this moment the best choice. If *C. dubia* is used, EPA recommends that a shortened version of the 7-day test, referred to as the 4-day test (Masters et al. 1991), should be used. The 4-day test is shorter than the standard 7-day short-term test, which makes it attractive for the TIE investigation, however, it is significantly less sensitive (Oris et al. 1991).

In our opinion, three test methods with *C. dubia* should be evaluated prior to fractionation depending on the extent of chronic toxicity in the whole sample. If the chronic response in the whole sample is not pronounced (e.g., NOEC between 25 and 50% effluent dilution), the 7-day static-renewal test should be used. If the NOEC is between 5 and 25% the abbreviated version of the standard chronic test, or 4-day test can be used. If the chronic toxicity is below the NOEC of 5%, the effluent and its fractions are likely to show acute toxicity, and, therefore, a static acute 48-hour test can be used.

After choosing the appropriate test method, the investigator is faced with limited fractionation procedures that can be applied to remove the source of chronic toxicity, particularly when the 7-day test is used. For example, during this study an artificial toxicity was observed in the eluate samples from ion exchange columns. Exchange resins like cation, anion, or clinoptilolite are composed of finely sized polymer or natural mineral beads that serve as an insoluble support or matrix for attached (covalently bonded) functional groups. The functional groups are ionic. The functional group ion is bonded to the resin, while other ion(s), of the same charge are exchanged. The cationic exchange resins effectively remove metals, calcium and magnesium and adds sodium. Therefore, basic macroelements like Ca, Mg, Na, and K are added back to the eluate using reagent grade chemicals, e.g., $NaHCO_3$, $CaSO_4$, $MgSO_4$ and KCl. As stated above, the concentration of both divalent and monovalent ions and their ratio in aquatic systems play a significant role in biochemical processes of various organisms. The eluate from the exchange column does not always have a ratio typical for freshwater, therefore, changes resulting from these manipulations can affect the performance of daphnids during short-term tests.

Finally, while evaluating results from both TIE studies, one critical question was frequently discussed. Are we ready to interpret the results from chronic TIE programs? The chronically toxic effluents are usually discharged from relatively efficient and sound treatment systems with an average of 95 percent removal of particulate matter, metals or biochemical oxygen demand. Therefore, to accommodate removal of chronic toxicity usually large investments are necessary, only to discover that the chronic toxicity disappears within the initial mixing zone.

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